Fabrication and Characterization of a Silk-PCL Based Scaffold for Ligament Graft

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Certificate

This is to certify that the report entitled, **"Fabrication and Characterization of a Silk-PCL Based Scaffold for Ligament Graft"** submitted by Mr. **Shendge Aadeshkumar Suresh**, Roll no.: **211BM2014**, M. Tech, Department of Biotechnology & Medical Engineering, National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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Shendge Aadeshkumar Suresh

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ABSTRACT

Ligament gets damaged very often in cutting and pivoting sports. Current gold standards for ligament replacement are based on autografts which has limitations of inadequate strength and donor site morbidity. Thus, ligament tissue engineering is promising strategy for replacing severely damaged ligaments beyond repair. The objective of current study is to fabricate a hybrid scaffold for ligament graft with high tensile strength which will support cell proliferation. Briefly, knitted silk scaffold was made by use of wild type silk from *Antheraea mylitta*. Polycaprolactone (PCL) was electrospun on these knitted scaffold to facilitate cell growth. Degradation study and mechanical testing of scaffolds were carried out at five time points (0, 3, 7, 14, 21 d). Mouse fibroblasts (L929) were cultured on these nano-micro scaffolds to investigate the cell adhesion and proliferation potential. Cell proliferation was visualized under fluorescent microscopy and was analysed by MTT assay. Hybrid scaffolds showed slow degradation rate and high tensile strength, 22.75 ± 0.43 N at end of day 21. Cell adhesion efficiency was determined to be 92.28±0.61%. L929 cells grew profusely on the hybrid scaffold as confirmed from fluorescent microscopy and MTT assay. Silk-PCL based hybrid scaffold promises to be a better platform for ligament tissue engineering with optimal biocompatibility and mechanical properties.

Keywords -: Ligament tissue engineering, Electrospinning, *Antheraea mylitta*, Polycaprolactone, Knitted scaffold

CHAPTER 1

INTRODUCTION

1. Introduction

Anterior cruciate ligament (ACL) is a ligament in knee joint which connects femur and tibia. The role of ligament is to shift loads between bones on both sides of ligament. Ligament keeps the both bones in place in joints by proper alignment and avoids dislocation. Ligament tissue is composed of ligament fibroblast embedded into extracellular matrix. Ligament tissue is mostly made up of mostly extracellular matrix with low density of fibroblast. Ligaments have very limited requirement for nutrients and oxygen. When ligament experiences more forces than it can bear, permanent damage will be caused to ligament. Limited supply of nutrient and oxygen provide very limited capacity for healing and regeneration. Thus medical intervention is necessary in case of damage to ligament. Tissue engineering is promising approach for ligament tissue repair and regeneration.

Currently different approaches are being tested in various laboratories. In recent years, integration of newly made ligament tissue with bones is found to be a limitation. Integration of ligament with bone is recent focus for ligament tissue engineering work. Combinations of different suitable biomaterial with properties are being used for fabricating competent scaffold.

1.1. Anterior Cruciate Ligament

Anterior cruciate ligament is the cruciate ligament of knee. Knee joint consist of four ligaments, anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), medial collateral ligament (MCL) and lateral collateral ligament (LCL), as shown in fig 1.1. The anterior cruciate ligament is present on anterior side of knee. The anterior ligament limits forward motion of femur with respect to tibia in knee joint. The anterior cruciate ligament is dependable for stabilizing turning movements at the knee that takes place during

cutting and pivoting activities. It gets injured more than any other ligament. ACL injuries are most common ligament associated injuries in cutting or pivoting sports such as football, rugby etc. Healing potential of ACL is very limited as compared to other ligaments of knee.

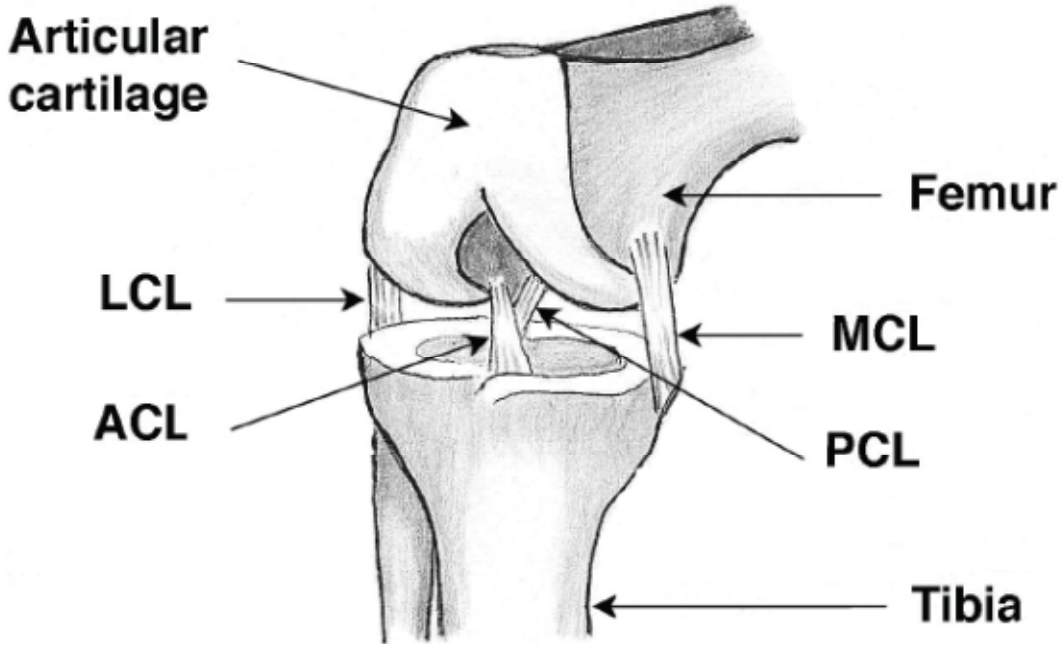


Fig. 1.1 Anatomy of knee joint

1.2. Tissue engineering

Tissue engineering is a combination of cells, materials, and suitable biochemical factors to replace biological functions in an effort to improve clinical procedures for the repair of damaged tissues and organs. It is a combination of biological principle and engineering principles. Tissue engineering approach uses biodegradable and biocompatible biomaterials with sufficient structural and mechanical properties to imitate the organization of the native tissue.

Tissue engineering is a multidisciplinary approach to the production of living tissue in vitro and in vivo that proposes innovative solutions for the treatment of organ and tissue damage. Tissue engineering has gain a great interest for replacement or repair of musculoskeletal system. Tissue engineering approach is currently being used in musculoskeletal system for cartilage, bone, ligament and tendon.

1.3. Scaffold

In tissue engineering, scaffold act as template for growth of cells to form functional tissue or organ. It is assumed that, cell only be them self cannot form the tissue or organ. Cells need a preformed structure template or scaffold to form tissue or organ. Cells adhere to the scaffold and micro-environment in scaffold allows cells to proliferate. Scaffold provide appropriate environment for cell growth and proliferation. Scaffold should able to provide suitable properties for graft implantation such as high mechanical strength and interconnected channel. Ideally scaffold should provide support for cell interaction, cell proliferation and cell differentiation. Scaffold should be biocompatible. The biodegradation of scaffold should be compared to that of native tissue. Scaffold should provide strength for neo tissue and during initial period of post-implantation. Scaffold should be able to provide versatile application for different modification.

Over past century, various biocompatible materials have been used for implantation. Different materials such as polymers, ceramics and metals have been investigated. Polymeric biomaterial provides flexibility for the scaffold which advantageous in some tissue.

1.3.1. Silk

A variety of insects and spiders produces silk. Silk is fibrous material made-up of two different proteins, sericin and fibroin. Fibroin act like core of filamentous structure while

sericin functions as glue. Silk serves for different purpose for different insects. It has been used in versatile way in nature. Different type of silk shows different type of properties. Many insects are shown to produce more than one type of silk. Functions of silks shows varied functional range from web structure and catching prey, safety line to cocoons for protection. Silkworm silks are contains mainly of fibroin, while the chief protein of spider silks is spidroin. Silk has been used for medical application as sutures for centuries. Currently it's being exploited as a biomaterial for scaffold in tissue engineering application. Silk from most common silkworm *Bombyx mori* have been extensively studied. It has shown potential in biomedical application. In recent years, wild type silk has been of great interest. Wild type silk have shown better cell adhesion than mulberry type silk. More extensive studies are needed for exploration of true potential of wild type silk in tissue engineering application.

Ideally scaffold should provide support for cell interaction, cell proliferation and cell differentiation. Scaffold should be biocompatible. The biodegradation of scaffold should be compared to that of native tissue. Scaffold should provide strength for neo tissue and during initial period of post-implantation. Scaffold should be able to provide versatile application for different modification.

1.3.2. PCL

Polycaprolactone is linear aliphatic polyester. PCL shows a slow biodegradation. In human body, PCL is degraded by hydrolysis of ester linkage. It is biocompatible, bioresorbable synthetic polymer. PCL is available in different molecular weight and influence its properties. Thus provide diverse option for suitable properties. It has been approved by Food and drug administration. It has been extensively used in slow drug release device and suture material. PCL do not have any isoform as that of PLA thus PCL shows uniformity with materials. PCL is hydrophobic materials. Hydrophobic nature

of PCL avoids cell attachment. The material can be modified to allow cell adhesion and proliferation.

1.4 Electrospinning

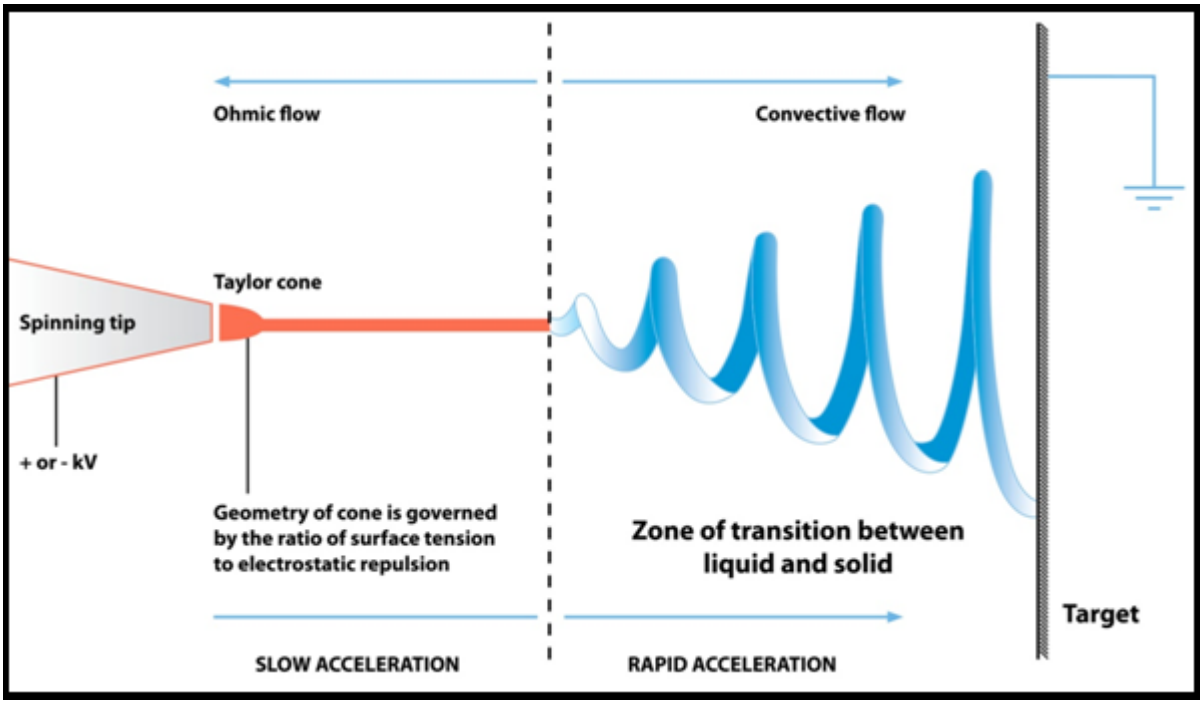


Fig 1.2 Flow in electrospinning. Diagram shows formation of Taylor's cone ohmic flow and convective flow during elctrospinning

Electrospun fibers are formed after applying a high voltage (Potential difference) between a liquid solution and a collector. A polymeric solution is used for the elctrospinning. When high voltage is applied, polymeric solution overcome surface tension and forms a cone. This cone is called as Taylor's cone. The solution kept in syringe will flow through needle and forms nano-fibers. This nano-fiber gets accelerated toward oppositely charged collector. The collector collects the fibers. When a stationary collector is used, fibers will form a 'mesh' of randomly aligned fibers.

As shown in figure 1.2 after application of voltage Taylor's cone will be formed at spinneret. The flow can be divided into two types of flows, ohmic flows and convective flows. It depends on the voltage applied. Elctrospinning depends upon various parameters. Parameters such as applied voltage, temperature, humidity, solution viscosity etc. can affect morphology and structure of fibers. Electrospinning has been used extensively for tissue engineering application. Electro-spun fibres resemble as that of extra cellular matrix of tissue, specially like collagen.

CHAPTER 2

LITERATURE REVIEW

2. Literature Review

2.1 Anterior Cruciate Ligament

The anterior cruciate ligament (ACL) of is one of the strongest ligament. Anterior cruciate ligament consists of extracellular matrix (80%) and fibroblast (20%). Extracellular matrix is mainly consists of collagen with small amount of elastin, proteoglycan and glycoprotein[1]. Collagen present in ACL is mainly of type I. Small amount of collagen type III is also present.

Ligament acquires tensile strength and elasticity mainly from triple-helical type collagen type I. Collagen molecules organizes themselves into parallel fibrils. Nano scale fibrils assemble to form micro scale fibers. The fibers are aligned in parallel direction to the load i.e. along with major axis of the ligament. Diameter of the micro scale fibers increases with increasing age[2, 3]. Ligament fibroblasts interact with each via connexion 32 and 43 positive gap junction made by extended cell processes. ACL injuries occur mostly at the interface between ligament and bone. Interface at femoral site is more prone to get injured than interface at tibial-site[4, 5]. ACL has shown to have very limited healing potential, which intensify need of therapeutic involvement.

2.1.1 Current treatment and its limitation

Biological tissue autograft reconstruction using the patellar tendon has become the most popular procedure in surgical treatment of a ruptured ACL. Autologous hamstring are progressively utilized for ACL reconstruction due to donor site morbidity associated with bone-patellar tendon-bone grafts (BPTB) [6]. Capability to integrate with bone via its bony ends makes the BPTB graft gold standard. Moreover, it possesses intact insertion sites that can serve as functional transitions between ligament and bone. The tendon grafts have to

be fixed mechanically within the bone tunnel. The graft-to-bone interface is not formed as the native interface site, instead nonmineralized soft tissue is found in bone tunnel[7]. Thus graft fixation at the tibial and femoral tunnels, instead of the isolated strength of the graft, represents the weakest point during the early postoperative healing period[8]. The surgical procedures for ACL replacement are found be associated for pain, muscular weakness or knee instability. Outcomes after anterior cruciate ligament reconstruction have been disappointing[9, 10].

2.2 Silk

Silk is a natural protein fibre produced by insects and spiders. Silk consist of two measure protein, fibroin and sericin. Fibroin is the fibrous protein while sericin acts as 'glue'. Initial immunogenic activity of silk was traced back to sericin, thus will be eliminated from silk for its use as biomaterial.

Silk has been used in medical application for more than 3000 years. Silk has shown excellent biocompatibility. Silk fibres have shown to provide high tensile strength as compared to other materials. Tissue engineering applications such as ligament tissue engineering needs fibrous material with high tensile strength. Thus Silk can be used efficiently as scaffold material for ligament tissue engineering.

Silk is broadly categorised into two types

- Mulberry Silk
- Non-mulberry Silk (Wild type Silk)

Mulberry silk can obtained from *Bombex mori*, which feeds on mulberry leaves. It has been cultivated at large scale due to its important in textile industry. This silk has been exploited over years for biomedical application.

Other type of silk produced from different insect are referred collectively as Non-mulberry or wild type silk. This group consist of different types of silk. In India, three different kind of wild silk are found Tasar silk, Muga silk and Eri silk. Wild types of silks are still being investigated for exploiting its entire potential.

2.2.1 Tasar Silk

Tasar silk is subdivided into Tropical Tasar and Oak Tasar. Tropical tasar is produced by *Antheraea mylitta* while Oak tasar is produced by *Antheraea proyeli*. Tropical tasar is produced only in India. Production of Tasar has increased by 36% in year 2011-12 over previous year (Central silk board Annual Report 2011-12). Developing additional application of tasar silk in tissue engineering application will help to develop its market value.

Antheraea mylitta silk has been used in very few tissue engineering studies. Still now all the studies have been reported from same research group. Kundu et al used tropical tasar silk as substrate for in vitro growth cella. They have studied its potential as scaffold in case of articular cartilage tissue engineering [11]. They have also used tasar silk based scaffold for cardiac tissue engineering. *Antheraea mylitta* silk found to be better than the *Bombex mori* silk [12]. These results may be due to presence of RGD sequence.

2.2.2 Use of silk as biomaterial in Ligament Tissue engineering

Various groups have already used mulberry silk as biomaterial for various type of tissue engineering work. Goh et al (2008) have described use of mulberry silk based scaffold for the ligament tissue engineering in their various research papers. Hybrid Scaffolds were prepared by lyophilizing silk fibroin solution on knitted silk to form micro-porous sponge. The scaffolds were seeded with mesenchymal stem cells. Cell showed abundant deposition

of collagens, prominent component of ligament extra cellular matrix[13]. This hybrid scaffold shown to provided suitable environment for proliferation of ligament fibroblast[14]. Slow degradation of in vivo implanted scaffold has been shown. In recent study, they have shown tri-lineage culture of fibroblasts, bone marrow stem cells and Osteoblasts on hybrid silk scaffold. They co-cultured cells on different scaffold and joined them with stitches[15]. Differentiation of bone marrow stem cells into fibrocartilage lineage has been confirmed with gene expression study by RT-PCR. Chen X et al used knitted silk scaffolds incorporated with collagen. In vitro studies showed higher expression of collagen than only silk scaffold[16].

Silk fibroin can be used in different forms such as woven, braided, knitted and non woven. The features of each type have been summarized into table 2.1.

	Woven	Knitted	Braided	Non-Woven
Composition	Yarn	Yarn	Yarn	Fiber
Formation	Interlace	Inter loop	Intertwine	Bond or entangled
Porosity	High	Very high	High	High
Mobility	Limited	Tremendous	Limited	Very slight

Table 2.1 Different type of scaffold fabrication methods

2.3 PCL

Polycaprolactone (PCL) is biodegradable polyester. PCL is slowly degrading aliphatic polyester. It has been to show that degradation of PCL is about only 2% after 110 weeks [17]. PCL has been approved for implantation for few applications by the Food and Drug Administration [18]. PCL have shown adaptability for scaffold preparation for different

methods. Various methods such as Electrospinning [19, 20] and fused deposition modelling [21] have been used widely for fabrication of scaffolds.

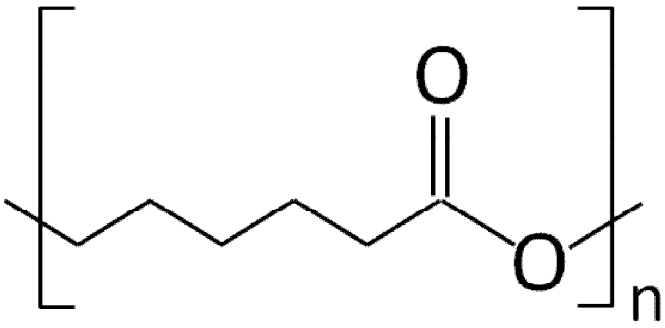


Fig 2.1 Chemical Structure of PCL

PCL has been used as scaffolds in many tissue culture applications. PCL has been used in Nerve tissue engineering, Bone tissue engineering etc. In case of ligament bone interface tissue engineering, PCL has been used in combination of other biomaterials such as PGA [22], chitosan[23]. Slow degradation of PCL provides time for tissue restoration. Use of electro-spun fibers with knitted silk scaffold holds the cells on scaffold and allows the cells to multiply and adhere to silk fibers.

2.3.1 Electro-spinning of PCL

PCL can be fabricated by different methods such as electrospinning, gravity spinning[24], phase separation, solid freeform fabrication[25]. Electrospinning of PCL has particular advantage as the morphology of electro-spun fibrous mesh resembles as that of extra cellular matrix. High surface area of PCL allows better attachment of cell on the mesh[26]. Electrospinning of PCL has been performed with different solution system. Different solvents such as chloroform, methanol, hexafluoroisopropanol (HFIP), tetrahydrofuran (THF), dimethylformamide (DMF) and dichloromethane (DCM) have been utilized for electrospinning.

Use of diverse solvents and their combinations are extensively studied over the years. The morphology and uniformity of fibers highly depends on the use of solvent. Supaphol et al (2006)[27] used combination of DCM/DMF for preparation of PCL solution. DCM/DMF has been used in ratio of 1:1. Concentration of PCL used is 12%. Similarly Mavis et al (2009)[28] used combination of DCM/DMF at ratio of 1:1 for preparation of 8 % and 12% solution of PCL. Different operating parameters have been used for electrospinning of PCL. Use of operating parameter depends on the solution parameter such as viscosity, conductivity etc. The parameters of the different elctrospinning setup have to be optimised separately.

CHAPTER 3

OBJECTIVES

3. Objectives

Recent development in the area to tissue engineering and regenerative medicine has made possible to regeneration and restoration of musculoskeletal tissue which is under high stress. The regeneration of tissue requires scaffold that provides similar properties of that of native tissue. Novel biomaterials have been investigated for tissue engineering. Among the novel materials which have been utilized recently wild type silk seems to be promising for ligament tissue engineering. This study investigates use of this novel biomaterial for the ligament tissue engineering.

Objectives of current research work are:

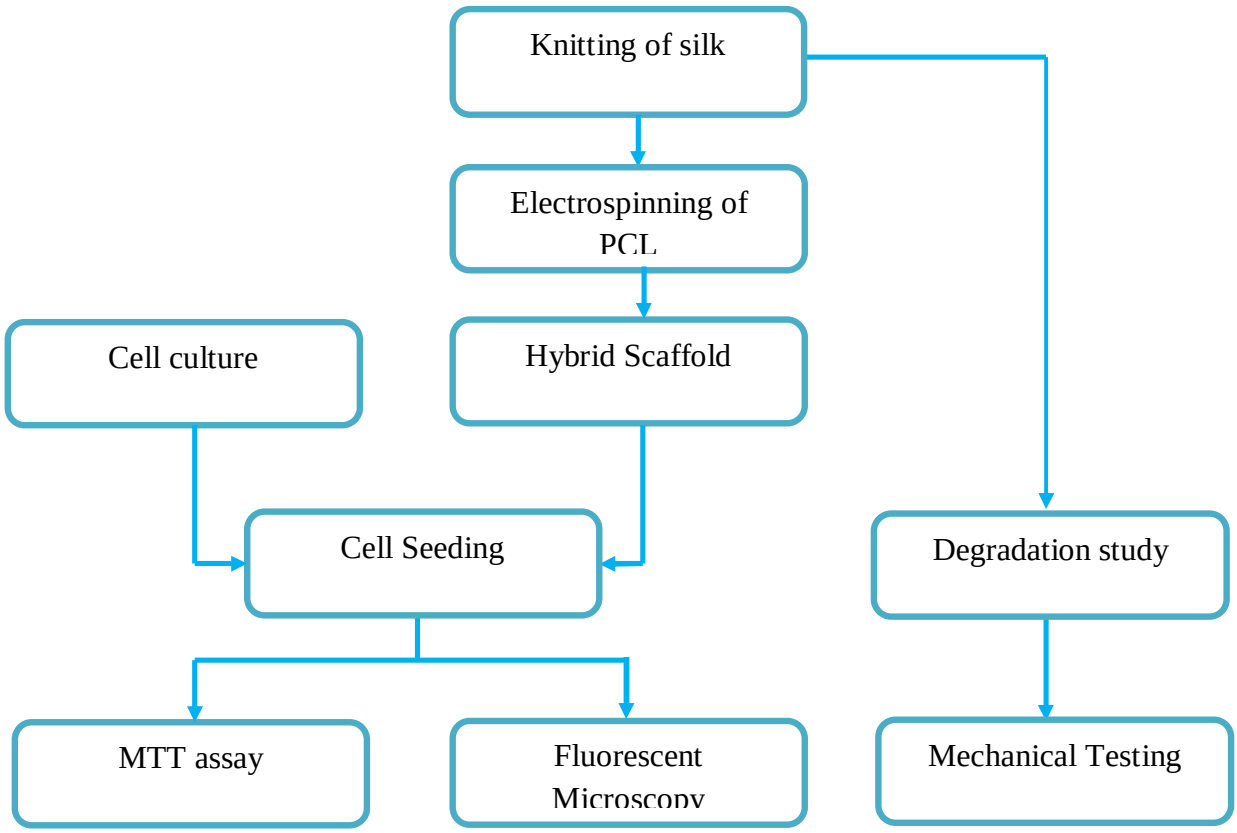
1. To fabricate the knitted-electrospun scaffold with wild type of silk produced by *Antheraea mylitta* and polycaprolactone for ligament tissue engineering.
2. To study degradation and tensile strength of knitted silk scaffold. The scaffold should able provide similar properties that of native ligament.
3. To investigate cells adhesion and biocompatibility of hybrid scaffold by culturing cells on scaffold. For successful use of scaffold in tissue engineering it must be biocompatible.

CHAPTER 4

PLAN OF WORK

Plan of Work

Wild type silk provide novel biomaterial for the scaffold. The hybrid scaffold with knitted silk and electrospun PCL mesh was fabricated. Hybrid scaffold was tested for biocompatibility by in vitro cell culture. The present project work was carried out by following work plan.



CHAPTER 5

MATERIALS AND METHODS

5. Materials and Methods

5.1 Fabrication of scaffold

5.1.1 Preparation of Silk Yarn

Silk was procured from Raw Tasar Silk Depot, Chiabasa, Jharkhand, India. Different types of silk fibroin were purchased. The Silk yarn fibroin having 12 fibres were used to make yarn. By using 10 fibroin yarns were prepared. Yarn was made with 120 filaments. These yarns were slightly twisted to give approximately circular cross-section.

5.1.2 Preparation of knitted silk Scaffold

Knitting was carried out by using Brother Knitting Machine (Model no. KH830).Nine needles were used to make scaffold. Use of nine needles on machine provides sufficient space to make scaffold of 20mm width. The parameters for knitting were optimized for the yarn. For the yarn with 120 filaments tension was adjusted to 4. Long scaffold were made to minimize effect of pulling force on initial knitting and to obtain uniform scaffolds.

Fig. 5.1 Brother Knitting Machine (Model no. KH830) used for knitting of silk scaffolds

Briefly, knitting was carried out pushing five alternate needles were pushed to ‘Out’ position. The yarn was loaded to K-carriage and pulled to right end. All five needles got shifted to ‘Working’ position. The ‘Claw’ weights were used to give uniform pull for knitting process. Remaining four needs were pushed to ‘Working’ position. K-carriage moved to and fro from right end to left end with counting. Knitting was finished by cutting the yarn about 10 cm away from last needle and then K-carriage was moved over knitted silk. Knitted silk was collected and cut in to 6 cm pieces. They were loaded on wire frame immediately to avoid any damage to knitting.

The scaffolds were cut at distance of 60 mm with closing the ends with silk fibres.

5.1.3 Loading of scaffolds on wire frame

The scaffolds were loaded on wire frame made by aluminium wire of diameter 1mm to keep scaffold in planar conformation. The frame was made about same size that of scaffold. The scaffolds loaded on the wire frame were used for elctrospinning. Figure 5.1 shows scaffold load on the wire loop.

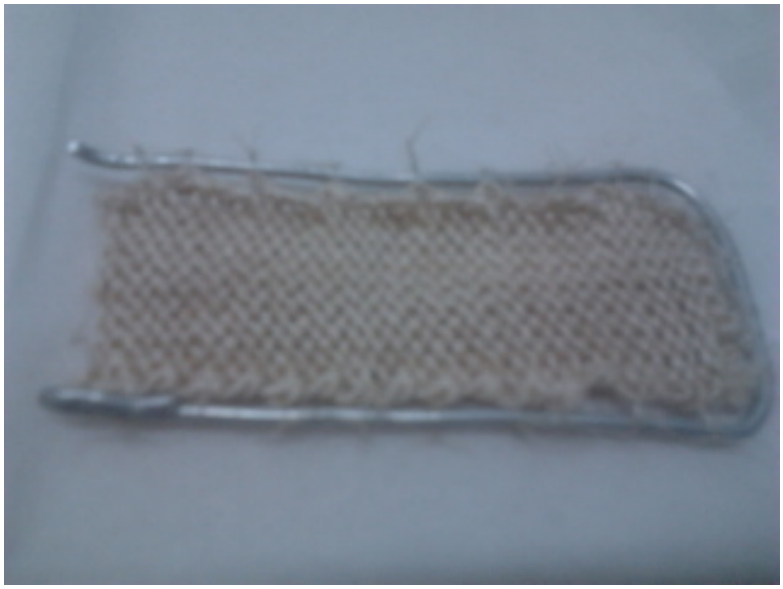


Fig. 5.2 Knitted scaffold loaded on wire loop

5.1.4 Morphological characterization of knitted scaffold

The morphology of the knitted fibers was analyzed under optical scanning electron microscope. A JEOL JSM- 6480LV SEM was used in the experiment for morphology of fibers at an accelerating voltage of 20 KV. Scaffolds were cut into small square about 1 cm side. Scaffolds were mounted on the sample holder with the help of carbon tape. Each sample was coated with a thick layer of platinum by a JEOL JFC -1600 auto fine coater. The operating parameters were maintained at 20 mA for 90 seconds.

5.2 Preparation of PCL Mesh

5.2.1 Preparation of PCL solution

Poly (ε-caprolactone) [Molecular weight Mn=80,000] was purchased from Sigma-Aldrich. It is available in pellet form. The solvents to dissolve the PCL were selected after the literature review. Dichloromethane and Di-methyl formamide were used to make solution of PCL. The solvents were purchased from Merck Co. and used without further purification. Solutions for electrospinning were prepared in the quantity of 5 gram (wt). The chemical being used are toxic. All the solution preparation was done in fume hood. Polymer concentration of 10% wt/wt was used for electrospinning. For preparation of solution 0.5 gram of PCL pellets was weighted in small glass bottle. After that equal amount of dichloromethane and dimethylformamide were added to make final solution of 5 gram. The solution was kept for stirring on magnetic stirrer for 24 hours at low rotation speed. The glass bottle was capped and sealed properly. The solution was used for elctrospinning within 2 days of preparation of solution.

5.2.2 Electrospinning of PCL

The electrospinning setup was used for carrying out electrospinning of 10% PCL solution.. The parameters were fixed from previous work or by trial method. The solution was loaded into 5 ml glass syringe. Needle with inner diameter of 0.7 mm was used for electrospinning. Syringe was fixed with a syringe pump which allows controlled continuous flow of solution. Flow rate was maintained at 2 mL/hr. Distance between the tip of needle and collector was fixed to 10 cm. This distance was adjusted by rotating screw attached to collector stage. The collector plate was checked to make it horizontally levelled. Aluminium foil was kept on the stage. The Aluminium foil was connected to high voltage supply. A high voltage of 12 kV was applied between needle and collector.

Process Parameter	Values used
Distance between Tip and collector	10 cm
Applied voltage	12 kV
Flow rate	2 ml/hr

Table 5.1 Parameters used for electrospinning

Knitted scaffolds, which were previously made, were used for collection of electrospun mesh. As mentioned earlier, scaffolds were loaded on wire frame. For elctrospinning, two scaffolds were kept on the collector. Positions of scaffolds were such that they are not directly under need nor too far from needle. Scaffolds were moved intermediately to maintain uniformity in mesh formation. Scaffolds were flipped after one hour of electrospinning and kept for one more hour. After electrospinning for total two hours, scaffold were removed and used for biocompatibility testing.

5.3 Degradation and Mechanical Testing of Scaffold

Mechanical strength of construct is most important property for the ligament tissue engineering. Degradation of scaffolds was studied by keeping them in phosphate buffered saline at 37°C with pH 7.4. The pH was measured regular interval and maintained by addition of 1N HCl or 1N NaOH, if necessary.

Scaffolds degradation was analyzed based on ultimate failure load. Scaffolds were tested by use of TA-HD Plus Texture Analyser**.** Gauge length is set at 20mm for all scaffolds. The cross head speed is kept at 10 mm/min. The ultimate failure load determined for all the type of scaffold at day 0, 3, 7 and 14.

Fig. 5.3 TA-HD Plus Texture Analyser

5.4 Cell Culture

Cells were obtained from National Centre for Cell Sciences (NCCS), Pune. Mouse fibroblast cell line L-929 was obtained for its fibroblast nature and wide acceptance. The cells were cultured on DMEM/D-12 media**.** Powdered media was purchased for Hi-Media. It was reconstituted with 800 ml of double distilled water. 1.2 gms of Sodium bicarbonate

was added to media. It was then made to one litre. pH was adjusted to 7.2 with 1N NaOH and 1N HCl. Media was then filtered with filtration unit by using nitrocellulose membrane of 0.2µm pore size.

Media was supplemented with 5% serum and Antimicrobial -antifungal agents. Cells were cultured in T75 flask**.** Approximately 10 ml of media was used in every flask. The media was changed after every second day. Cells were sub-cultured after about 80% confluence or at 7^{th} day. The distribution ratio of 1:4 was used, as recommended by NCCS, Pune. The cells from third passage were used for seeding on scaffolds.

5.5 Biocompatibility Testing

5.5.1 Scaffold sterilization

Scaffolds were kept in separate $100mm^2$ Petri-plates. Scaffolds were sterilized with formaldehyde vapours for one hour and kept in bio-safety cabinet for 24 hrs to remove traces of formaldehyde vapour. The sterilised scaffolds were washed with 10 ml of sterilized PBS for three times. 10 ml of culture media was added to scaffolds to neutralize. After 24 hr, media was removed and scaffolds were used for cell seeding.

5.5.2 Cell seeding

Cells were seeded at concentration 2.5 X 10^6 cells/ml. 1 ml of cell containing media was used to seed the cells on scaffold. After 1 hr, 10 ml of DMEM/D-12 media supplemented with 5 % of serum was added.

5.5.3 Cell Adhesion Study

For cell adhesion studies scaffolds with 60mmX20mm were used (n=3). Cells were seeded on scaffolds. After 24 hrs, scaffolds were removed from Petri-plates. Media was collected in 15 ml centrifuge tube. The petri-plates were treated with 2 ml of trypsin to remove cell

attached to plate's surface. Trypsin with cells was transferred to tube containing media from petri-plates. Tubes were centrifuged at 4000 rpm for 15 min to separate cells from the media. The media was decanted. 1 ml of fresh media was added to pellet. Cell count was performed with haemocytometer. To find number of cells adhered to scaffold, number of cells from pellet were subtracted from initial number of cell seeded. Cell adhesion was expressed in terms percentage of cells adhered to scaffold.

5.5.4 MTT Assay

MTT assay was carried out be adding MTT reagent to the cell seeded scaffold. It was incubated for 4 hrs in presence of 5% CO_2 at 37°C. To dissolve formazan DMSO was added. Absorbance was taken at 630nm. Standard graph for MTT assay was plotted by adding MTT reagent to known cell densities.

5.5.5 Staining of cells with ethidium bromide

Stock solution of ethidium bromide of 2mg/ml was prepared. 100µl of ethidium bromide was added to 10 ml of cell culture medium for staining cells. Cells were incubated for 20 minutes with ethidium bromide. Further washed with PBS and observed under fluorescent microscope.

CHAPTER 6

RESULTS AND DISCUSSION

6. Result and Discussion

6.1 SEM analysis of scaffold

Knitting process involves use of knitting machine. Silk yarn may get damaged during process. Damaged scaffold will show lower tensile strength. To analyze damage to silk fibers SEM images were examined, as shown in figure 6.1. Knitted silk scaffold have not shown any type of damaged caused by knitting. In case of ligament tissue engineering, it is very important to have full strength of scaffold. Surface of the filaments provide information about intensity degumming process. Properly degummed silk fibres appear smooth. The yarn appears to smooth, suggesting silk has been properly degummed.

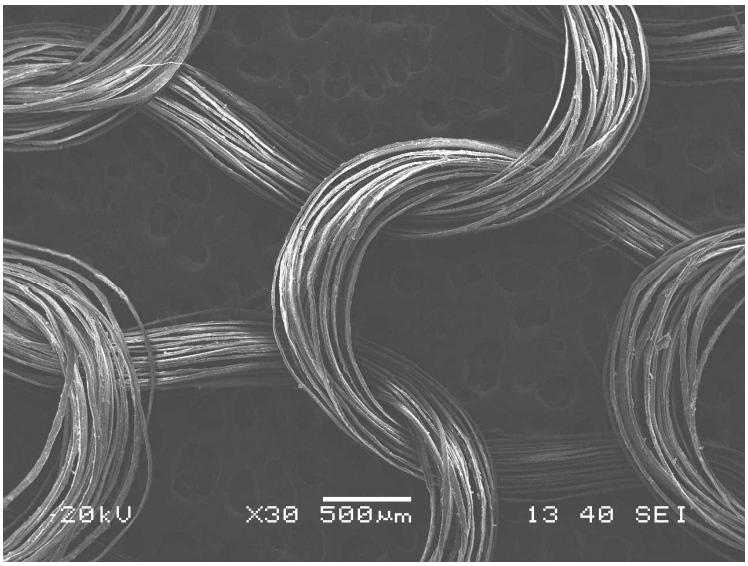


Fig. 6.1 SEM image of knitted silk scaffold

Electro-spun mesh of PCL was also analysed by SEM. Figure 6.2 shows the morphology of electrospun PCL mesh. It shows non-uniform distribution of fiber diameter. The fiber diameter is in range of 0.1 µm to 1 µm. The fibers resemble that of collagen morphology which forms assembly of similar sizes. The mesh of PCL provide good surface for cells to for attachment.

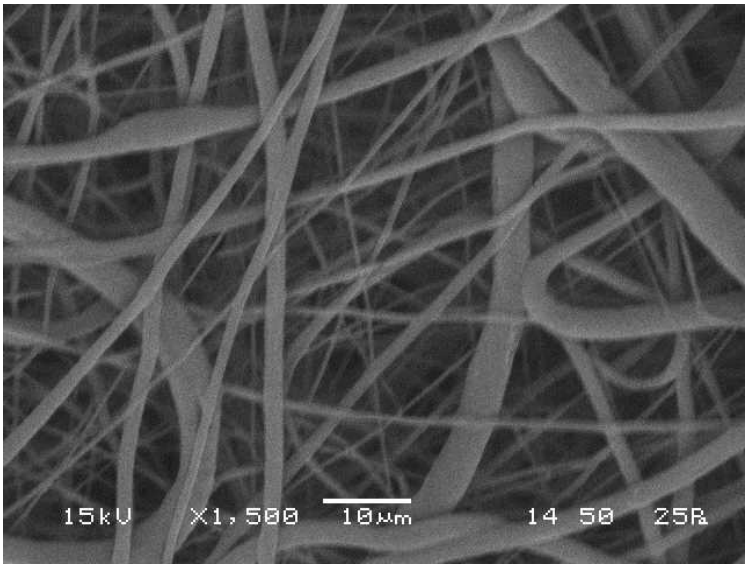


Fig. 6.2 SEM image of electro-spun PCL mesh

6.2 In vitro degradation of silk scaffold

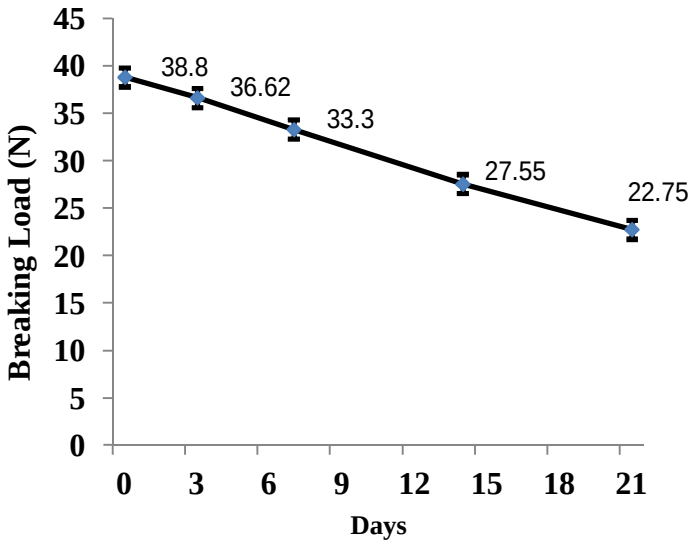


Fig. 6.3 Mechanical testing of scaffolds for ultimate tensile strength of knitted silk scaffold at five different time point

The tensile strength of silk scaffold decreased with degradation of silk over study period. The decrease in tensile strength of silk is found to be high as compared to PLGA[29]. Silk scaffold showed loss of 41.36% tensile strength over the period of 21 days. This decrease

in strength is slower than many other which were used previously. Knitted silk scaffold will able to provide high tensile strength over the long period. The strength of knitted silk scaffold could be improved by using thicker yarn. Use of thicker yarn will reduce area for the secreted extracellular collagen fibers. Collagen and silk, both are among the strongest natural fibers. Using the minimal yarn diameter to provide required strength will be advantageous as it will allow deposition and direction orientation of collagen.

6.3 Cell seeding and proliferation assay

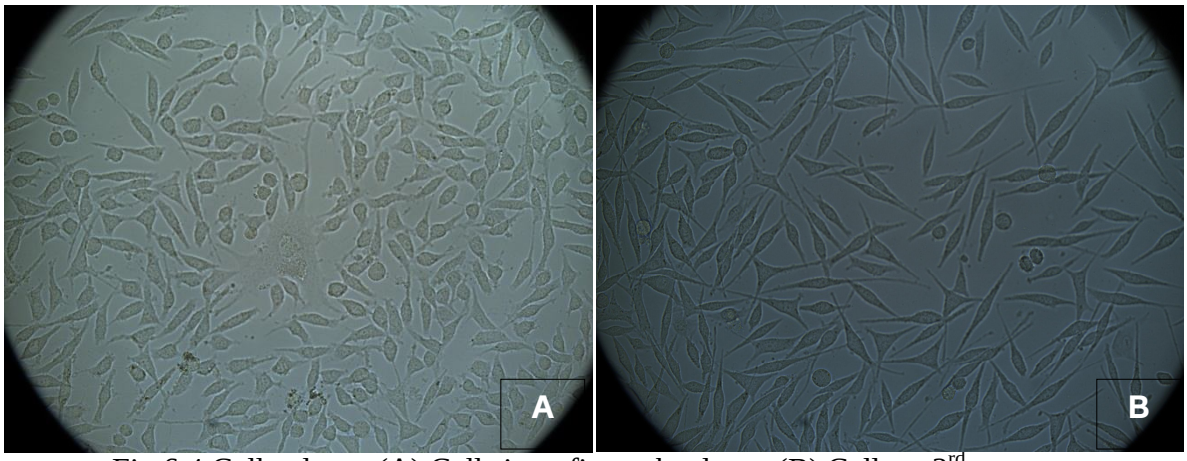


Fig 6.4 Cell culture. (A) Cells in at first subculture. (B) Cells at 3rd passage

Mouse fibroblast L929 shows the elongated morphology during the cell culture. The cells at first subculture are shown in fig 6.4A while 6.4B shows cells in third passage. Cells from third passage were seeded on scaffold.

Cells seeding efficiency is found to be 92.28 % ± 0.611. The cell seeding efficiency is found to better than scaffold[29]. High cell seeding efficiency allows overlooking of other method of cell seeding such as fibrin glue for cell seeding on scaffold. The cell seeding efficiency high may because of high surface area provided by electrospun PCL mesh. This mesh allows adherence of cells on scaffold.

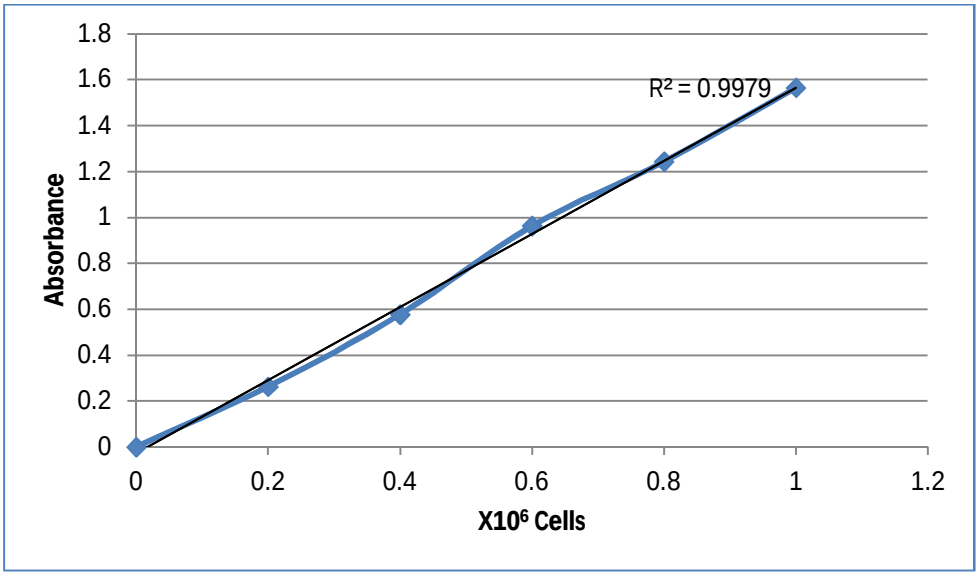


Fig. 6.5 Standard curve of MTT assay

Mouse fibroblast has shown proliferation on silk-PCL scaffold. Initially 1 X 10^5 cells were seeded on scaffold for proliferation assay. After day 3, number cells quantified by MTT assay is 2.7 X 10^5 cells. And after day 7, cell estimated to be 9.3 X 10^5 cells. MTT assay shows proliferation of cells on silk-PCL scaffold. This scaffold found to be suitable for cell growth.

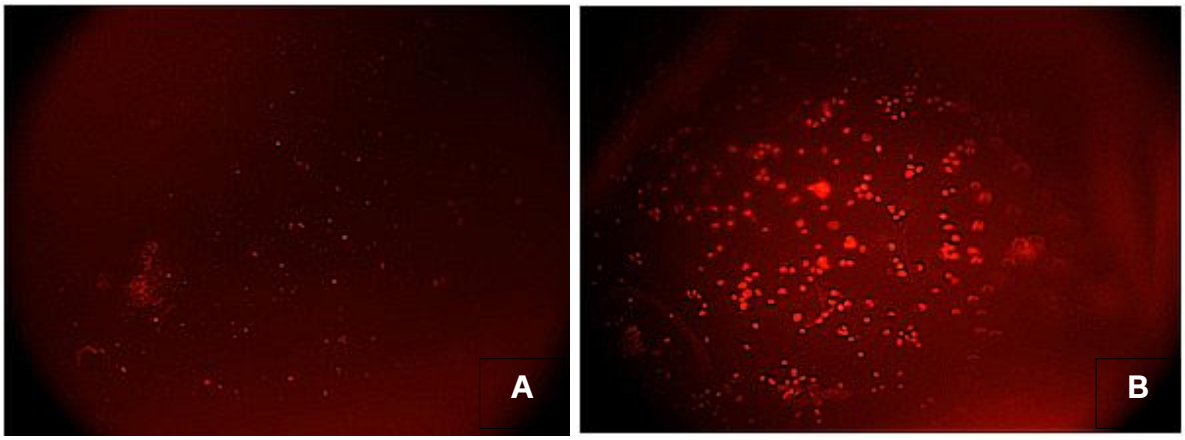


Fig 6.6 Cells stained with ethidium bromide, observed under fluorescent microscope at day 3 (A) and day 7 (B)

Fluorescent microscopy images taken by staining cell nucleus with ethidium bromide supports the result obtain from the MTT assay .The fluorescent microscopic images show

proliferation of cells from day 3 to day 7. Very few cells are visible in the day 3 image. In day 7 image proliferated cells can be observed clearly. The silk-PCL scaffold provides suitable microenvironment for growth of mouse fibroblast L929. Use of this biocompatible scaffold in ligament tissue engineering provides required tensile strength with high cell seeding efficiency.

CHAPTER 7

CONCLUSION

7. Conclusion

This research work for first time demonstrated use of wild type of silk produced from *Antheraea mylitta* for ligament tissue engineering. Wild type of silk have gain great interest as a biomaterial for tissue engineering in recent years.

The research also demonstrated successful use of knitted silk scaffold with electrospun PCL mesh. This combination of materials has been studied for first time. Results show that combinations of knitted silk with PCL mesh provides competent substrate for cell attachment and growth.

Ligament is one of the most tensile stress bearing tissue in the body. Tissue engineered scaffold should provide the tensile strength during healing. The degradation study of silk has shown slow loss of tensile strength. After 21 day of *in vitro* degradation, Silk scaffolds retained 60% of initial strength. Thus silk can provide the strength during *in vitro* cell culture and *in vivo* during early period of post- implantation.

The use of silk provides strength to scaffold while PCL mesh allows higher adhesion of cells. Results have shown 92.28 ± 0.61% cell adhesion efficiency. The wild type silk is allows better adhesion of cells due to presence of RGD sequence. This RGD sequence is absent in the mulberry silk. The competent adhesion of cells to scaffold eliminates need of any special cell seeding method. The results have shown that this hybrid scaffold supports proliferation of cells in vitro.

Thus from this study it can be concluded that hybrid scaffold made with wild silk and PCL is biocompatible and shows desirable properties for ligament tissue engineering. Use of silk-PCL scaffold in ligament tissue engineering will improve properties of ligament graft.

CHAPTER 8

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8. References

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